Intestinal Absorption of Tritium-Labelled Folic Acid in Idiopathic Steatorrhea:

Effect of a Gluten-Free Diet

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ABSTRACT

The intestinal absorption of folic acid in patients with idiopathic steatorrhea was studied by the oral administration of tritium-labelled folic acid in a dosage of 15 μg./kg. Results were expressed as a percentage of the orally administered folic acid radioactivity excreted in the urine over 24 hours. The mean excretion of radioactivity in 38 normal subjects was 48.2 ± 16.6% (mean ± SD), whereas eight patients with untreated idiopathic steatorrhea excreted only 16.7 ± 3.4% (mean ± SE).

The ability of the gluten-free diet to correct this absorptive defect was demonstrated by the finding of normal values in 11 patients in complete clinical remission for periods exceeding six months after institution of the diet. Serial studies in individual patients indicated that a significant improvement was obtainable in as short a period as two weeks following exclusion of gluten from the diet.

IMPAIRED intestinal absorption of folic acid was first reported in 1953 by Girdwood.[1] In a group of six patients with idiopathic steatorrhea the urinary excretion following an oral test dose was consistently low when compared with that of control subjects.

Subsequent reports by Girdwood,[2] Cox *et al.*,[3] and Chanarin, Anderson and Mollin,[4] while confirming these results in most respects, showed that a small minority of cases of idiopathic steatorrhea had a folic acid excretion index within the normal range. It was suggested by these authors that the impairment in folic acid absorption in idiopathic steatorrhea correlated poorly with the degree of steatorrhea and that folic acid absorption alone could not therefore be used as an index of the severity of the condition.

In 1950, Dicke[5] described the beneficial effects of excluding gluten from the diet of children with celiac disease. French, Hawkins and Cooke[6] have subsequently reported that most, if not all, patients with adult idiopathic steatorrhea respond to a gluten-free diet, although this sometimes requires up to six months or longer. The response in their patients included a return to normal of the blood picture, in some cases without the addition of vitamin B_{12}, folic acid or iron.

Despite this clinical evidence of an improvement in folic acid absorption following treatment with a gluten-free diet, Doig and Girdwood[7] reported the return to normal of folic acid absorption in only one of five patients with idiopathic steatorrhea in remission on a gluten-free diet for at least six months. Cox *et al.*[3] found a similar return to normal in only one patient out of six. On the other hand, four cases of infant celiac disease in remission on a gluten-free diet were studied by Girdwood and all showed normal absorption of folic acid.[8]

All of these earlier studies on the handling of folic acid were carried out by means of microbiological assay techniques, several of which are available.[8] For example, in Girdwood's[1] study, patients and control subjects were given 5 mg. of folic acid intramuscularly and urine was collected for 24 hours. An oral dose of 5 mg. of folic acid was then given and the urine collection was repeated. The amount of folic acid present in the urine was determined by microbiological assay (*S. faecalis*) and the values were expressed in terms of a ratio (the "excretion index").

These microbiologic methods suffer from several disadvantages such as the occasional development of mutations of the test organism, inhibition of growth in the presence of samples containing antibiotics or folic acid antagonists, contamination of the test medium with folic acid–active substances or with other bacteria, and lack of specificity.[8] It was felt therefore that a more simple and specific assay method might be of value and in 1960, tritium-labelled folic acid was introduced by two groups of workers for the study of folic acid metabolism in man.[9, 10] Studies with this material confirmed the existence of a defect in the absorption of folic acid in idiopathic steatorrhea.[9, 11, 12]

The following communication reports the results of further investigations with tritium-labelled folic acid in idiopathic steatorrhea, with particular attention devoted to the following points: (1) whether malabsorption of folic acid is universally present in idiopathic steatorrhea in relapse, and (2)

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whether an improvement in folic acid absorption takes place as a result of treatment solely with a gluten-free diet.

MATERIALS AND METHODS

1. *Preparation of tritium-labelled folic acid*

Tritium-labelled folic acid was prepared by an exchange reaction with tritiated acetic acid or with tritium gas as previously described[13] or was purchased from the Radiochemical Centre, Amersham, England. Purification was carried out by column chromatography on DEAE-cellulose, activities after purification ranging from 12 to 200 μc./mg. The major impurities in all preparations were p-aminobenzoylglutamic acid and two unidentified pteridines. The purified tritium-labelled folic acid was diluted with non-labelled folic acid to a specific activity of 12 μc./mg. The diluted preparation was then dissolved in sufficient normal saline containing 0.2% $NaHCO_3$ to give a stock solution containing 200 μg. of folic acid/ml. The stock solution was stored in the dark at 5° C.

An oral dose of 15 μg./kg. was used in order to facilitate comparison with previous studies carried out by our own and other groups on the handling of intravenously administered folic acid at this dose level.[9,10]

2. *Oral administration of tritium-labelled folic acid*

In order to eliminate the effect of possible tissue unsaturation, all subjects were preloaded with non-labelled folic acid. Preloading greatly affects both urinary excretion and plasma levels of folic acid after an oral dose, as can be seen from Figs. 1 and 2. In order to determine whether a single preloading dose was sufficient to eliminate errors from this source, the urinary excretion of tritium-labelled folic acid was measured in a control group of 11 normal subjects following preloading with a single intravenous injection of 30 mg. of non-labelled folic acid (Folvite, Lederle) given 30 minutes before ingestion of the test material; the test was repeated after seven days in which a daily intramuscular dose of 30 mg. of folic acid had been given. The urinary excretion of radioactivity after the single intravenous preloading dose was 53.6 ± 5.0% (mean ± SE) and that after the multiple preloading doses was 57.0 ± 5.1% (mean ± SE). This difference is not significant and therefore the more convenient single intravenous preloading dose was adopted.

Subjects were fasted overnight and the measured dose of tritium-labelled folic acid (15 μg./kg.) was given orally in 100 ml. of distilled water. Fluids were encouraged throughout the collection period and the subject was allowed to resume a normal diet after three hours.

Although the major part of the excretion of radioactivity occurred in the first six hours, urine was collected for 24 hours.

3. *Assay of urine radioactivity*

Urine, 0.5 ml., was pipetted into a 20-ml. glass counting-vial (Wheaton Glass Co., Milville, N.J.) and evaporated to a volume of *ca.* 0.1 ml. in an oven at 80° C. Hyamine (prepared by the method of Eisenberg[14]); 0.4 ml., was then added, followed by 1 ml. of ethanol and 5 ml. of a scintillator solution (toluene containing 0.6% 2,5-diphenyloxazole (DPO) and 0.02% of 1,4-bis-2(5-phenyloxazolyl) -benzene (POPOP) (Pilot Chemicals Inc., Watertown, Mass.). Quadruplicate determinations were carried out on all urine samples.

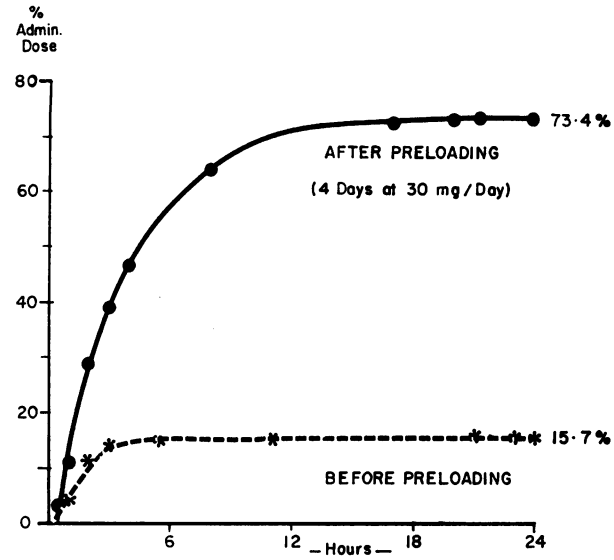

Fig. 1.—Cumulative 24-hour urinary excretion of radioactivity after the oral administration of 1 mg. of tritium-labelled folic acid (*ca.* 15 μg./kg.) before and after preloading.

As an alternate method, 1 ml. of urine was added to a counting-vial containing 3 g. of naphthalene (reagent grade, Matheson, Coleman and Bell). Fifteen millilitres of a scintillator solution was then added, made up as follows: 77 ml. dioxane, 23 ml. ethanol, 25 mg. POPOP, and 1 g. DPO. The samples were

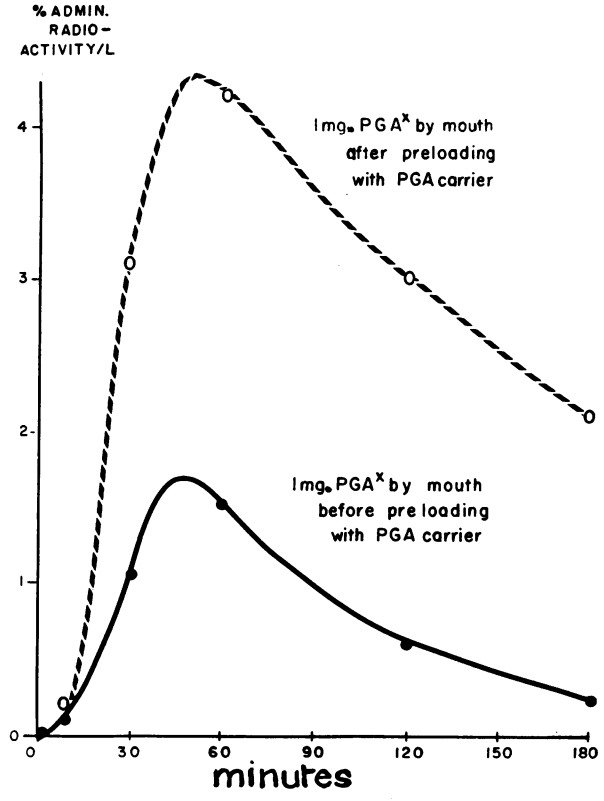

Fig. 2.—Plasma folic acid radioactivity after the oral administration of 1 mg. of tritium-labelled folic acid (*ca.* 15 μg./kg.) before and after preloading.

counted at —5° C. Efficiencies averaged 7.2%. If the deep-freeze temperature were raised, the addition of ethanol to this scintillator solution could be omitted with a consequent improvement in efficiency. The latter method was not suitable for concentrated urines containing a heavy sediment because of the absorption of folic acid on the insoluble residue at the bottom of the counting-vial, and consequent low counts.

4. *Counting methods*

A Packard TriCarb Liquid Scintillation Counter was used for all tritium assays. The samples were counted with a discriminator window setting of 10-100, voltage at Tap 6, and a deep-freeze setting of —5° C. Background was 60-80 counts per minute. At least two 10-minute counts were made on all samples. Because of the variation in the degree of quenching encountered with varying concentrations of urine, all samples were recounted after the addition of an internal standard (tritiated toluene, New England Nuclear Corp., Boston, Mass.). Efficiencies ranged from 5% to 14%. Results were expressed as a percentage of the orally administered folic acid radioactivity excreted in the urine over 24 hours.

5. *Subjects*

Thirty-eight control subjects ranging in age from 23-80 years were selected from ambulant and convalescent patients. These control subjects showed no evidence of hematologic or gastrointestinal disorders and were free of infection and wasting disease. All were in a good state of nutrition. The hemoglobin level exceeded 13.5 g. % in males and 12.5 g. % in females. The urine was free of significant sediment and the blood urea nitrogen was normal.

Fifteen patients with idiopathic steatorrhea were selected for study. The diagnosis in all cases was confirmed by the usual methods, including chemical fat balance and peroral jejunal biopsy. Eight of this group were studied while the disease was active, and before institution of any therapy. Eleven patients were studied who were in complete clinical remission on a gluten-free diet; the remission had extended from six months to three years. Serial measurements of folic acid excretion were obtained in five patients untreated and again at intervals of two weeks and six months after institution of a gluten-free diet.

6. *Identity of radioactive compounds in the urine*

It was considered of interest to determine in a typical case of idiopathic steatorrhea how much of the urinary radioactivity was due to unchanged folic acid, and how much to labelled breakdown products.

Chromatographic analysis of the radioactive compounds in the urine was therefore carried out. One mg. each of p-aminobenzoylglutamic acid, folinic acid, folic acid, and 2-amino-4-hydroxy-6-methylpteridine was dissolved in 1 ml. of 0.2% sodium bicarbonate. The solution was added to 10 ml. of urine from a patient with idiopathic steatorrhea in relapse after the oral administration of 15 μg./kg. of tritium-labelled folic acid (14 μc.). The urine was diluted to a volume of 120 ml. with distilled water and applied to a 1 x 7 cm. column of DEAE-cellulose. A flow rate of 0.4 ml. per minute was used and the effluent was collected after being passed through a recording ultraviolet absorptiometer. After

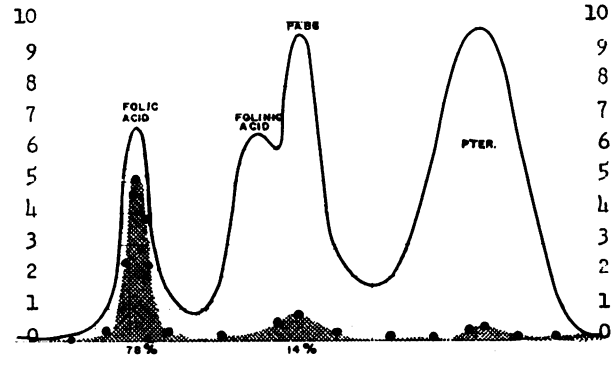

Fig. 3.—Distribution in urine of radioactivity displaced by non-labelled folic acid (30 mg.), as determined by column chromatography on DEAE-cellulose. The upper line shows ultraviolet absorption of the effluent as the added marker compounds (2-amino-4-hydroxy-6-methylpteridine, p-aminobenzoylglutamate, folinic acid, and folic acid) are eluted from the column. Fractions were collected manually and counted; the lower shaded area shows the distribution of tritium radioactivity in the effluent. The percentage of administered radioactivity present in the individual peaks is also shown. Urine was collected for 24 hours after the flushing dose.

application of the diluted urine, the column was developed with phosphate buffers of 0.01, 0.03, 0.06, 0.2 and 0.4 M. at pH 6.9. At the end of the run, the column was cleared with 0.5N sodium hydroxide solution. The location of the carriers in the collected fractions was determined from the absorptiometer record, identification being aided by determination of diazotizable amine in the various fractions before and after reduction by the Bratton-Marshall Method.[15] The chromatograph record illustrating the distribution of radioactivity is reproduced in Fig. 3.

It will be noted that most of the radioactivity (78%) was recovered in the form of unchanged folic acid; 14% was present as paraminobenzoyl-glutamic acid. No tritiated water was present.

RESULTS

Normal subjects.—The excretion of radioactivity in 38 normal subjects ranged from 18.8% to 80.9% of the dose with a mean of 48.2 ± 16.6% (mean ± SD). Fig. 4 demonstrates the distribution of excretion values in the subjects studied.

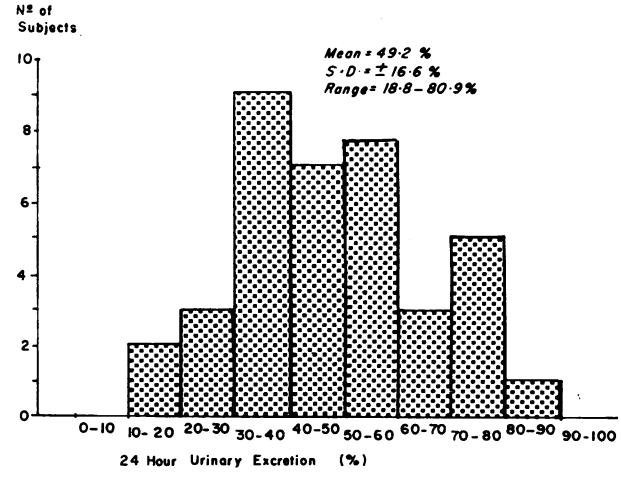

Fig. 4.—Distribution of radioactivity excretion values in 38 normal subjects after oral administration of 15 μg./kg. of tritium-labelled folic acid.

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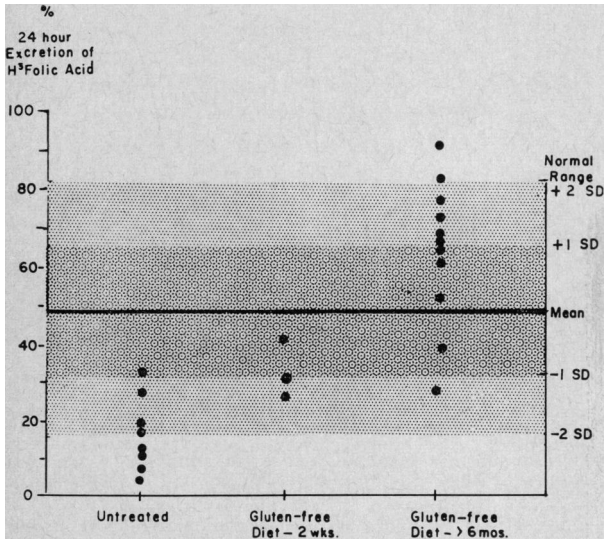

Fig. 5.—Twenty-four hour urinary excretion of radioactivity after oral administration of 15 μg./kg. of tritium-labelled folic acid to patients with idiopathic steatorrhea before and after treatment with a gluten-free diet. The shaded areas represent the normal range of excretion values.

Idiopathic steatorrhea in relapse.—Eight patients untreated and in relapse showed a mean excretion of 16.7 ± 3.4% (mean ± SE) (range 6.0-32.6). Only four of the eight patients in this group gave values which overlapped the normal range; the mean excretion was significantly less than that of the control group ($P<0.001$) (Fig. 5).

Idiopathic steatorrhea after six months on a gluten-free diet.—Eleven patients in complete clinical remission on a gluten-free diet for periods exceeding six months showed a mean excretion of 64.8 ± 5.5% (mean ± SE) (range 27.9-93.2), which is higher than in the control group. Indeed, two individuals showed excretion well above the normal range (Fig. 5).

Serial studies in idiopathic steatorrhea in relapse and following institution of a gluten-free diet.—Three patients were studied untreated and in relapse and again after two weeks' treatment on a gluten-free diet. During this period there had been cessation of diarrhea and a beginning weight gain.

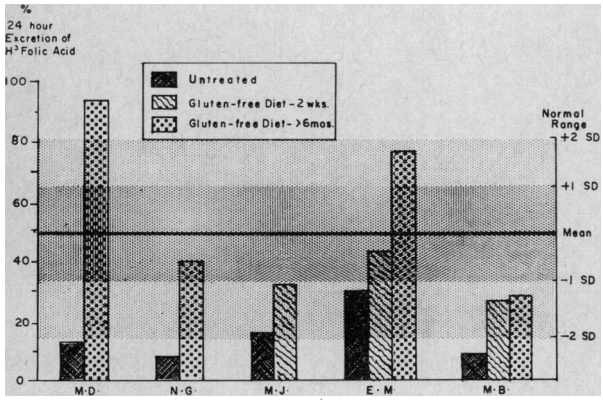

Fig. 6.—Serial measurements of urinary excretion of radioactivity after oral administration of 15 μg./kg. of tritium-labelled folic acid to individual patients with idiopathic steatorrhea before and at intervals after institution of a gluten-free diet.

The mean excretion for this group was 17.2 ± 6.3% before and 33.5 ± 4.5% (mean ± SE) after institution of a gluten-free diet. The difference between the means is significant ($0.01<P<0.02$) (Fig. 6).

Four patients were studied untreated and in relapse and then after six months of treatment with a gluten-free diet when all were in complete clinical remission. The mean excretion for this group was 13.4 ± 5.1% before and 59.4 ± 15.6% (mean ± SE) after the six-month period. The difference between the means is significant ($0.02 < P < 0.05$) (Fig. 6).

DISCUSSION

The demonstration of impaired intestinal absorption of fat is mandatory for the diagnosis of idiopathic steatorrhea. The absorption of other nutrients, however, such as iron, calcium and vitamin B_{12}, although usually impaired, is not consistently so. Furthermore, the degree of malabsorption of these other entities correlates poorly with the degree of steatorrhea.

From the present study it is apparent that the latter observations also apply in the case of folic acid. Four of our eight patients in relapse showed an impaired absorption of folic acid, the remaining four overlapping the normal range. Furthermore, there was no correlation between the degree of folic acid malabsorption and the actual amount of steatorrhea. The measurement of folic acid absorption by this technique, therefore, is of diagnostic significance when low values are encountered and as such merits consideration as a screening test for intestinal malabsorption; values within the normal range, on the other hand, do not exclude this diagnosis. It is probable that those patients with impaired absorption of folic acid would eventually develop megaloblastic anemia if left untreated for a long enough period.

Improved absorption of folic acid was found in all our cases of idiopathic steatorrhea which were restudied after treatment with a gluten-free diet. In three of our patients, this improvement was significant even after as short a period as two weeks on a strict gluten-free regimen, as all three attained values within the normal range during this period. From the clinical standpoint, our patient group demonstrated a dramatic response to this dietary treatment with cessation of diarrhea, beginning weight gain and a sense of well-being often manifest within several days after gluten exclusion. Although follow-up fat balances were not carried out, the objective improvement in folic acid absorption correlated quantitatively with the clinical remission.

After periods of clinical remission on a gluten-free diet exceeding six months, all 11 patients in this study showed normal folic acid absorption values. The mean value of this group was actually at the upper limit of normal. These observations are in contrast to those of Doig and Girdwood,[7]

and Cox *et al.*,[3] who reported a return to normal of folic acid absorption in only one of five patients and one of six patients, respectively. We have yet to encounter a case in which a low value in relapse did not return to normal following clinical remission induced by a gluten-free diet. For this reason we are led to believe that this particular absorptive defect is reversible in most, if not all, patients by isolated dietary treatment and that the need for supplementary folic acid therapy in this disease is open to question.

Peroral jejunal biopsies were obtained in 14 of the 15 patients with idiopathic steatorrhea studied. In all cases the mucosa exhibited the characteristic blunting or loss of the normal villi along with a chronic inflammatory cell infiltration into the lamina propria, characteristic of the histologic alteration in this disease. Two of these patients were rebiopsied after six months' treatment with a gluten-free diet and, despite complete clinical remission and a return of folic acid absorption to normal, showed no change in the pathologic picture. An additional five patients in remission on the diet for periods exceeding six months, and in some cases up to three years, all showing normal absorption of folic acid, were biopsied and shown to have the characteristic mucosal atrophy and chronic inflammation. It is thus apparent that, in this small group, a clinical remission and correction of folic acid malabsorption by a gluten-free diet was not accompanied by any significant improvement in the histologic lesion of the intestinal mucosa.

Because most of the "folic acid" in the diet occurs in the form of polyglutamyl conjugates rather than as free pteroylglutamic acid, it is possible that studies on the absorption of folic acid alone do not give a true picture of the status of the absorption of total dietary folic acid-active substances. It is conceivable that in idiopathic steatorrhea an impairment of the ability of the gut to break down the naturally occurring folic acid conjugates to readily absorbable forms may occur. Since pteroyltriglutamate resembles more closely the conjugated natural forms of folic acid than does pteroylmonoglutamate, tritiated pteroyltriglutamate is being prepared and its absorption is now being studied in normal subjects and in patients with idiopathic steatorrhea. A study is also being carried out on normal subjects on the effect of variations in the gluten content of the diet on the absorption of folic acid.

SUMMARY AND CONCLUSIONS

Intestinal absorption of folic acid has been studied by measuring the urinary excretion of radioactivity following oral administration of folic acid labelled with tritium.

Four out of eight patients with idiopathic steatorrhea in relapse showed impaired intestinal absorption of folic acid.

A clinical remission induced by a gluten-free diet was associated with a return of folic acid absorption to normal.

In this study, correction of the absorptive defect for folic acid by this means was partial after two weeks of treatment and complete after six months.

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PAGES OUT OF THE PAST: FROM THE JOURNAL OF FIFTY YEARS AGO

PAIN AS A SYMPTOM IN PULMONARY TUBERCULOSIS

The referred pleuritic pains are important as they may easily lead to the wrong diagnosis. The most important are those referred to the shoulders and arms and often mistaken for rheumatism. The pain may sometimes be felt down to the wrist or hand. There is often a cutaneous and muscular tenderness and sensitiveness to pressure. The explanation of these localizations is probably the sympathetic connexions of the brachial plexus and also the fact that the first and second dorsal nerves form part of this plexus as well as giving off the first and second intercostals. The importance of these shoulder pains, which vary considerably in intensity, has been emphasized by Pottenger, who maintains that they are of great importance in early diagnosis, often being the first indication of disease in the chest, though this may not be tubercular. Another very important group of pains are those referred to the abdomen along the course of the lower intercostal nerves. When acute they may simulate an acute abdominal condition, especially when on the right side, the sharp pain, referred to the abdomen and the rigidity of the corresponding rectus being very misleading. An examination of the chest, in many cases of sudden abdominal pain, will reveal a pleurisy over the lower lobe posteriorly, which will explain the pain and obviate errors in diagnosis and treatment. It is not uncommon to find pleurodynia or neuralgia of the intercostal nerves associated with pleurisy. While this may and does occur during the course of pulmonary tuberculosis apart from signs of pleurisy, and may be caused by toxic absorption or other general condition, it is fairly frequently due to pleural inflammation. The finding of cutaneous hyperaesthesia and points of tenderness over the exits of the cutaneous branches of the intercostal nerves near the spine, in the axilla, or near the sternum, should demand an examination of the deeper structures.—Robert C. Paterson: *Canad. Med. Ass. J.*, 3: 782, 1913.